


Origins of CD4⁺ circulating and tissue-resident memory T-cells

Quynh P. Nguyen, Tianda Z. Deng,
Deborah A. Witherden and
Ananda W. Goldrath 

Division of Biological Sciences, University of
California San Diego, La Jolla, CA, USA

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Correspondence: Ananda W. Goldrath,
Division of Biological Sciences, University of
California San Diego, 9500 Gilman Drive,
La Jolla, CA 92093-0377, USA.

Email: agoldrath@ucsd.edu

Senior author: Ananda W. Goldrath

Summary

In response to infection, naive CD4⁺ T-cells proliferate and differentiate into several possible effector subsets, including conventional T helper effector cells (T_{H1}, T_{H2}, T_{H17}), T regulatory cells (T_{reg}) and T follicular helper cells (T_{FH}). Once infection is cleared, a small population of long-lived memory cells remains that mediate immune defenses against reinfection. Memory T lymphocytes have classically been categorized into central memory cell (T_{CM}) and effector memory cell (T_{EM}) subsets, both of which circulate between blood, secondary lymphoid organs and in some cases non-lymphoid tissues. A third subset of memory cells, referred to as tissue-resident memory cells (T_{RM}), resides in tissues without recirculation, serving as 'first line' of defense at barrier sites, such as skin, lung and intestinal mucosa, and augmenting innate immunity in the earliest phases of reinfection and recruiting circulating CD4⁺ and CD8⁺ T-cells. The presence of multiple CD4⁺ T helper subsets has complicated studies of CD4⁺ memory T-cell differentiation, and the mediators required to support their function. In this review, we summarize recent investigations into the origins of CD4⁺ memory T-cell populations and discuss studies addressing CD4⁺ T_{RM} differentiation in barrier tissues.

Keywords: protective immunity; T helper.

Introduction

CD4⁺ T lymphocytes are a key element of adaptive immunity, acting to direct and enhance functions of innate cells, B-cells and CD8⁺ T-cells in response to diverse pathogens. Following infection, naive CD4⁺ T-cells proliferate and have the potential to differentiate into at least seven functionally distinct T helper (T_H) subsets with unique effector functions within the circulation, secondary lymphoid organs (SLO) and infected tissues.¹ Depending on the type of immunological threat, early host–pathogen interactions result in an infection milieu that directs differentiation of naive CD4⁺ T-cells to acquire the helper functions to ensure that each class of pathogen is countered with the appropriate immune response.² Once infection has cleared, the majority of the effector cells die via apoptosis during a contraction phase, while a small proportion persists and differentiates into long-lived memory cells. This memory population enables a rapid and robust secondary response against recurring pathogens and, thus, is pivotal in conferring lasting cellular immunity particularly against pathogens where neutralizing antibodies alone are insufficient at providing long-term protection.

While significant advances have been made in understanding the generation and maintenance of memory CD8⁺ T-cells and B-cells, the molecular mechanisms underlying the generation of memory CD4⁺ T-cells remain relatively elusive. Two major obstacles have contributed to this knowledge deficit. First, CD4⁺ T-cells are inherently less proliferative and the CD4⁺ memory T-cell population appears to decline following antigen clearance, while the CD8⁺ memory T-cell population, if established, is typically stable^{3–5} and thus, fewer cells are available for study. Second, the existence of functionally distinct effector T_H cell subsets hinders the ability to characterize a common CD4⁺ memory T-cell precursor. Further, T_H effector and memory T-cells also exhibit significant plasticity and can interconvert between lineages, both *in vivo* and *in vitro*, adding an additional layer of complexity to identifying memory precursor cells in CD4⁺ T memory studies.^{6–11}

Memory T-cells have been conventionally divided into central memory (T_{CM}) cells, which circulate between the blood and SLO, and effector memory (T_{EM}) cells, which can migrate from the blood into non-lymphoid tissues^{1,11}. Over the past decade, evidence of a novel subset of memory

cells called tissue-resident memory T-cells (T_{RM}) has emerged. T_{RM} are seeded in non-lymphoid tissues, particularly at barrier sites like the skin and intestinal mucosa.^{12–15} T_{RM} have limited recirculation out of tissues and serve as sentinels at sites of potential reinfection, where they coordinate the initial response to pathogens and provide a substantial boost to tissue immunity via direct antigen recognition and recruitment of circulating immune cells.^{13,16} The majority of studies on T_{RM} have focused on $CD8^+$ tissue-resident lymphocyte differentiation, survival and function, while less is known about their $CD4^+$ counterparts, which are also known to mediate antiviral responses.^{17,18} The relationship between circulating and tissue-resident $CD4^+$ memory T-cell origins and T_H effectors subsets is currently unknown. A better understanding of the precursors of $CD4^+$ T_{RM} and the molecular mechanisms mediating their differentiation will allow us to harness the protective capacity of this memory population and modulate their activity in the context of infection or inflammatory diseases.

Despite the challenges in studying memory $CD4^+$ T-cells, efforts in recent years focusing on different aspects of memory development have begun to elucidate a more comprehensive picture for the generation and maintenance of memory $CD4^+$ T-cells. In this review, we summarize recent studies addressing the identity of memory $CD4^+$ T-cell populations and their precursors in both the periphery and non-lymphoid tissues.

$CD4^+$ T-cell memory in secondary lymphoid organs

Despite clear differences between memory $CD4^+$ and $CD8^+$ T-cell populations, including the range of effector cell heterogeneity,³ the models for memory $CD8^+$ T-cell formation have served as a useful framework for investigation of memory $CD4^+$ T-cells. During the primary response of antigen-specific cytotoxic $CD8^+$ T-cells (CTL), two effector $CD8^+$ T-cell populations can be identified based on surface expression of killer cell lectin-like receptor subfamily G member 1 (KLRG1) and interleukin-7 receptor- α (CD127).¹⁹ The $KLRG1^{hi}CD127^{lo}$ population, termed terminal effector cells (TE), is predominantly lost during the contraction phase, while the $KLRG1^{lo}CD127^{hi}$ subset contains memory-precursor cells (MP), which can differentiate into long-lived memory $CD8^+$ T-cells.¹⁹ $CD4^+$ T-cells also express KLRG1²⁰ and CD127.²¹ However, the roles of these molecules in memory $CD4^+$ populations are not well established nor are clear strategies for distinguishing shorter-lived effector and precursors of memory T_H populations.

Evidence for long-lived $CD4^+$ memory T-cells capable of responding to pathogen re-challenge has been documented in studies of adoptive transfer of T-cell receptor (TCR) transgenic T-cells^{22–25} and endogenous immune

responses.²³ However, the diversity of functional T_H phenotypes has made identification of distinct $CD4^+$ TE and MP effector populations challenging. Additionally, it is unclear whether all $CD4^+$ T_H effector T-cells possess the same potential to differentiate into long-lived memory cells. A separate MP may exist for each subset or there may be a unique effector subset with an inherent memory programme that can give rise to memory populations with the potential to generate T_H subsets with all or some effector functions [T_{H1} , T_{H2} , T_{H17} , T follicular helper cells (T_{FH}), T regulatory cells (T_{reg})] in a secondary infection. An elegant study by Tubo *et al.*²⁶ addressed this issue by following the differentiation of individual $CD4^+$ T-cells responding to infection. Utilizing over 80 distinct TCR clones that can specifically respond to *Listeria monocytogenes* (LM) infection, they demonstrated that all microbe-specific naive $CD4^+$ T-cells have the potential to give rise to memory cells following acute infection.²⁶ Different individual naive $CD4^+$ T-cells generated antigen-specific effector populations with varying frequencies of T_{H1} and T_{FH} effector cells. Notably, the relative frequencies of these subsets were preserved into the memory phase, suggesting that both T_{H1} and T_{FH} effector populations contain precursors of memory cells that retain their effector T_H characteristics (Fig. 1a). These data favour the idea that some $CD4^+$ memory cells are relatively lineage-committed; however, a range of expansion potential and plasticity among progeny was also observed, suggesting that not all $CD4^+$ memory precursor cells may be equivalent.

T_{H1} and T_{FH} $CD4^+$ memory T-cells

In efforts to address these questions, several groups have used lymphocytic choriomeningitis virus (LCMV) to characterize the response of adoptively transferred SMARTA (SM) cells, which have transgenic expression of an MHC Class II-restricted T-cell antigen receptor (TCR) specific for LCMV glycoprotein amino acids 66–77.^{24,25,27,28} Meanwhile, other investigators have studied the endogenous polyclonal response by utilizing the peptide-loaded major histocompatibility complex class II (pMHCII) tetramer-based approach to identify antigen-specific $CD4^+$ T-cells.^{22,23,25} During acute infection with LCMV-Armstrong, antigen-specific $CD4^+$ T-cells differentiate into two main helper subtypes in the spleen and lymph nodes: T_{H1} and T_{FH} . T_{H1} cells express the transcriptional regulator T-bet and are known for secreting their signature effector molecule, interferon gamma ($IFN\gamma$), while T_{FH} cells express Bcl6 and their hallmark surface molecule C-X-C chemokine receptor type 5 (CXCR5), which allows for homing to germinal centres to support B-cell responses. To explore the origins of T_{H1} and T_{FH} memory cells, investigators utilized fluorescence-activated cell sorting to isolate T_{H1} and T_{FH}

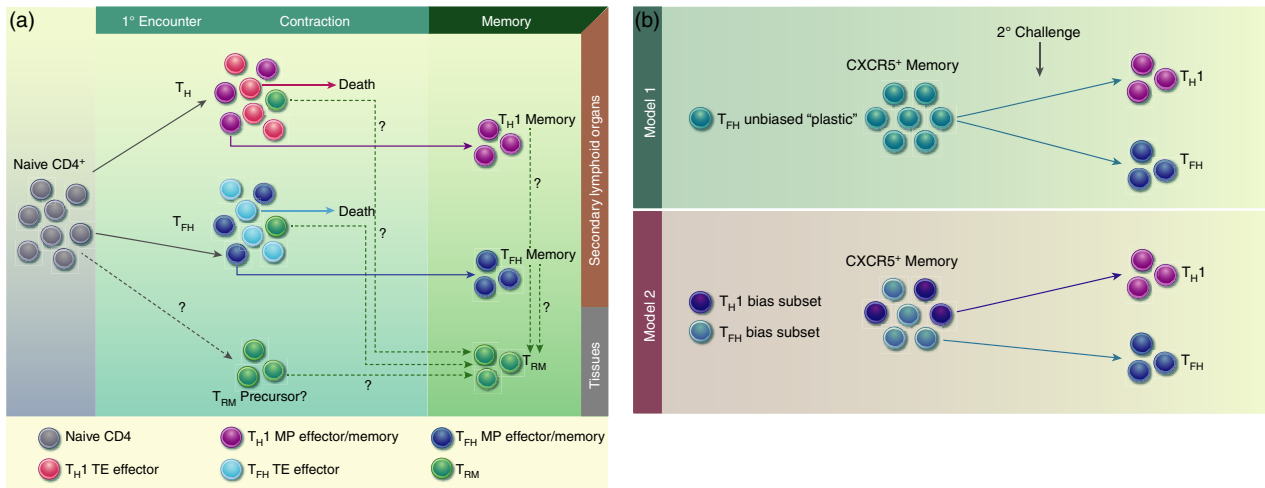


Figure 1. Models of CD4⁺ memory T-cell formation. (a) Upon antigen encounter, naive CD4⁺ T-cells differentiate into effector subsets based on the type of infection. Within each effector CD4⁺ subset, there potentially exist terminal effectors (TE) and memory precursor (MP) effectors. The majority of TEs die during the contraction, while MPs can survive and transition into resting memory cells. CD4⁺ tissue-resident memory cells (T_{RM}) may differentiate from: (1) the naive subset; (2) MP cells within the effector population; or (3) committed memory cells. (b) Two models for T follicular helper cell (T_{FH}) multi-potency: (1) T_{FH} memory cells retain cellular plasticity and can differentiate into T_{H1} or T_{FH} secondary effectors based on signals present during secondary challenge; (2) T_{FH} memory cells are actually a heterogeneous population with subsets that are biased or primed towards a particular secondary effector lineage (T_{H1} or T_{FH}).

effector and memory cells based on known markers, and studied their characteristics in the context of reinfection.^{21,24–26}

Marshall *et al.*²⁴ found that within the primary effector populations from the spleen at day 8, two CD4⁺ T-cell subsets that resembled the CD8⁺ TE and MP T-cells were observed. The TE-like population was marked by high expression of both P-selectin glycoprotein ligand-1 (PSGL-1) and lymphocyte antigen 6 complex (Ly6C), while the MP-like effector cells were PSGL-1^{hi}Ly6C^{lo}. In contrast to the PSGL-1^{hi}Ly6C^{hi} cells, the PSGL-1^{hi}Ly6C^{lo} MP-like population exhibited greater longevity in uninfected hosts, increased proliferation following antigen re-challenge, and similar gene-expression profiles with day 60 PSGL-1^{hi} memory CD4⁺ T-cells.²⁴ These results led the authors to propose that differential expression of Ly6C can distinguish TE from MP cells within the T_{H1} subset. At day 8, PSGL-1^{lo}Ly6C^{lo} effector cells showed high expression of known T_{FH} markers (ICOS, CXCR5, PD-1). This PSGL-1^{lo}Ly6C^{lo} subset was found along with PSGL-1^{hi}Ly6C^{hi} and PSGL-1^{hi}Ly6C^{lo} T_{H1} cells within the memory cells at day 150 after infection, suggesting that MP of both T_{H1} and T_{FH} phenotypes may persist long term.²⁴ Interestingly, while the PSGL-1^{hi}Ly6C^{lo} MP population was thought to be primarily T_{H1} cells, it was later shown by Choi *et al.*²⁵ that the PSGL-1^{hi}Ly6C^{lo} MP population actually contains both CXCR5[−] T_{H1} and CXCR5⁺ T_{FH} cells at comparable frequencies. These results highlight the complexity and heterogeneity within CD4⁺

memory T-cells and the need for further studies to fully understand the nature of the CD4⁺ memory T-cell pool.

To investigate the potential of T_{FH} memory cells for re-differentiation upon reinfection, Hale *et al.*²⁷ utilized expression of CXCR5 and Ly6C to distinguish between T_{H1} (CXCR5[−]Ly6C^{hi}) and T_{FH} (CXCR5⁺Ly6C^{lo} & CXCR5⁺Ly6C^{int}) memory populations following acute infection with LCMV-Armstrong, then transferred each of the three subsets into naive hosts for reinfection. T_{H1} memory cells mostly maintained high Ly6C expression with few effector cells gaining CXCR5 expression, while T_{FH} memory cells were able to give rise to both CXCR5[−]Ly6C^{hi} T_{H1} cells and CXCR5⁺Ly6C^{lo/int} T_{FH} cells. This multi-potency of T_{FH} memory cells during re-challenge has also been observed in acute bacterial infection with LM²⁹ as well as in viral influenza infection.²⁸

In a concurrent study, Pepper *et al.* addressed CD4⁺ memory T-cell differentiation using LM infection and the expression of CXCR5 and CC chemokine receptor 7 (CCR7), a marker used in previous studies to identify T_{CM}. During acute infection, antigen-specific effector cells segregated into a CXCR5[−] population favouring the T_{H1} phenotype and a CXCR5⁺ population.³⁰ A fraction of the CXCR5[−] T_{H1} population, which the authors termed T_{H1} effector memory cells, survived to a memory time point and, upon re-challenge, produced T_{H1} effector cells. The CXCR5⁺ effector population included cells with high expression of the lineage-defining factor Bcl6, were localized to follicles and were termed T_{FH}, while cells with

lower Bcl6 levels showed co-expression of CCR7 and were termed T_{CM} . It is worth noting that the T_{FH} subset resembled what some studies term germinal centre T_{FH} cells (GC T_{FH}); GC T_{FH} can lose expression of Bcl6 after infection, suggesting that, depending on the time point, the CXCR5⁺ population can include cells that did not enter the GC as well as those that were transiently in the GC. While both T_{FH} and T_{CM} in this study expressed CXCR5, T_{CM} were not seen in the follicles and, upon re-challenge, produced both T_{H1} effector cells and CXCR5⁺ cells that likely include T_{FH} and GC T_{FH} .³⁰ Notably, Choi *et al.*²⁵ found that precursors of T_{FH} or the CXCR5⁺ populations show greater potential to develop into memory cells compared with T_{H1} precursors and share gene-expression signatures with memory CD8⁺ T-cells. These results suggest that both T_{H1} and T_{FH} effector T-cells can give rise to memory cells, and CXCR5⁺ T_{FH} -derived memory cells have greater plasticity in generating secondary effector phenotypes.

Corroborative reports affirming the increased plasticity of T_{FH} memory relative to T_{H1} memory upon re-challenge suggest that T_{FH} memory populations may retain a greater cellular 'stem-ness' and are capable of providing a more comprehensive and robust secondary response during re-infection. Two possible models can explain the multi-potency demonstrated by CXCR5⁺ memory cells (Fig. 1b). One possible explanation is that T_{FH} memory cells are inherently more plastic compared with other T_H memory cells and, therefore, retain the ability to differentiate into alternative helper lineages upon reinfection. A second possibility is that the CXCR5⁺ memory population actually contains distinct subsets that are programmed or biased towards a specific T_H lineage upon secondary challenge. In this case, CXCR5⁺CCR7⁺ could distinguish memory cells with the greater potential for re-expansion, while CXCR5⁺CCR7⁻ cells may be long-lived T_{FH} /GC T_{FH} cells that have downregulated Bcl6 and PD-1, and are more similar to long-lived effector subsets. Based on the data currently available, neither hypothesis can be eliminated and further characterization of T_{FH} memory cells, perhaps using single-cell approaches, is needed to determine whether the multi-potency of T_{FH} memory is the result of cellular plasticity or population heterogeneity, or both.

In line with this idea, a recent study by Ciucci *et al.*³¹ utilized single-cell RNA sequencing to investigate the heterogeneity of antigen-specific CD4⁺ effector T-cells in response to acute LCMV infection. Visualization with t-distributed stochastic neighbour embedding (t-SNE) of day 7 effector T-cells yielded multiple transcriptionally distinct clusters showcasing the heterogeneity exhibited by T_{H1} and T_{FH} effector cells. At 30 days post-infection, single cell analysis also showed multiple distinct transcriptional clusters with shared T_{FH} features, supporting the idea that memory CXCR5⁺ T_{FH} multi-potency may be the result of population heterogeneity.

T_{H2} CD4⁺ memory T-cells

T_{H2} memory cells have been best characterized in the context of allergic inflammatory disorders,³² though some studies have highlighted this population's role in defense against parasitic worm infection. As mentioned previously, antigen-experienced CD4⁺ T_H cells contract more rapidly after pathogen clearance compared with CD8⁺ T-cells,⁴ which is why early investigations into T_{H2} memory relied on adoptive cell transfers of *in vitro* polarized T_{H2} effectors.³³ This system involved activating CD4⁺ T-cells *in vitro* with antigen and antigen-presenting cells (APCs) followed by culturing in T_H polarizing conditions³³ and subsequent adoptive transfer. Interestingly, *in vitro* generated T_{H1} and T_{H2} cells retained their expression of lineage-defining transcription factors (TFs), T-bet and GATA3, respectively, for months after transfer into naive hosts.³⁴ However, upon viral infection with LCMV, *in vitro*-derived T_{H2} memory cells were able to adapt a T_{H1} phenotype and persist as a 'hybrid' memory cell with combined T_{H1} and T_{H2} characteristics.³⁴ Utilizing a similar *in vitro* polarization system, Endo *et al.*³⁵ identified an interleukin-5 (IL-5)-producing subset of T_{H2} memory cells in the spleen that is primarily responsible for asthmatic symptoms, such as eosinophilic infiltration into the airway, airway hyper-responsiveness and mucus hyper-production in a murine model of T_{H2} -driven allergic airway inflammation. These studies provided early evidence of the potential existence of T_{H2} memory populations, but data demonstrating direct *in vivo* generation were lacking until recently.

A study by Hondowicz *et al.*³⁶ provided key insights into T_{H2} memory studying the endogenous allergen-specific CD4⁺ T-cells induced in response to house dust mite (HDM) inoculation. Using pMHCII tetramers to follow antigen-specific CD4⁺ T-cells, the authors showed an expansion of allergen-specific CD4⁺ T_{H2} cells in SLOs and the lung following intranasal HDM administration. Notably, this allergen system induces both antigen-specific T_{H2} and T_{FH} cells, analogous to the T_{H1} and T_{FH} response against LCMV-Armstrong. The allergen-specific memory pool in the SLOs consisted of CXCR5⁺ and CXCR5⁻ cells that also expressed CCR7⁺, consistent with the earlier observations that memory T-cells retain characteristics of T_H effector phenotypes. The authors further explored characteristics of allergen-specific T_{H2} resident in the lung, which will be discussed in the lung CD4⁺ T_{RM} section below.

T_{H17} CD4⁺ memory T-cells

Though not as extensively characterized as other helper subsets, memory T_{H17} cells have been documented in both humans and mice, primarily in the context of autoimmunity.³⁷ Early memory experiments using LM

infection showed that T_H17 cells existed only transiently following intranasal infection.²³ However, it is worth noting that LM may not be an optimal infection for T_H17 studies as it is an intracellular pathogen³⁸ and most efficiently induces T_H1 cells. Muranski *et al.*³⁹ reported on long-lived memory T_H17 cells but, similar to early T_H2 studies, these cells required *in vitro* polarization prior to transfer into host mice. In a recent study of dry eye disease, Chen *et al.*⁴⁰ utilized a pre-clinical murine model of autoimmune ocular disease, where mice were subjected to 14 days of environmental desiccating stress followed by rest in normal conditions for 14 days, and found disease-specific pathogenic memory T_H17 cells in both the inflamed site and draining lymph nodes. Two cytokines associated with CD4⁺ memory T lymphocytes, IL-7 and IL-15⁴¹ were shown to be crucial in the maintenance of these pathogenic T_H17 cells. Neutralization of these cytokines with topical application of anti-IL-7 or anti-IL-15 antibody decreased the number of T_H17 cells in both the conjunctivae and lymph nodes, offering a potential therapy for autoimmune disorders. One crucial caveat to note is that these 'memory' T_H17 cells were studied under the chronic inflammatory environment of autoimmunity, perhaps under prolonged or recurrent exposure to antigen; therefore, this population's identity as true resting memory cells remains uncertain.

Uncovering the origin and identity of resting memory or MP cells within a particular helper T-cell lineage will lay the foundation for future molecular studies into how each memory T_H lineage is uniquely regulated. However, in the next section, we will review two biological requirements crucial for memory formation that appear to be conserved across all T_H subsets.

Memory differentiation cues: TCR signalling and IL-2

A recent comprehensive review of studies aimed at resolving the signals required for CD4⁺ memory T-cell formation⁴² discussed the instructive signals both during the 'early priming' phase of initial antigen recognition and activation as well as at 'late-acting checkpoints' prior to contraction that play a role in the effector-to-memory transition. Much like the signals important for CD8⁺ memory T-cell generation, strengths of TCR and co-stimulatory signalling also have profound effects on memory T_H development.^{42,43} Recent results from Snook *et al.*⁴⁴ demonstrated that TCR signalling has a direct impact on T_H memory formation. Utilizing a panel of TCRs specific for the same viral antigen, the authors showed substantial variability in TCR signal strength, expression of IL-2-receptor alpha (CD25) and activation of downstream TFs across the CD4⁺ memory T-cell population.⁴⁴ TCR clones with stronger TCR signalling appear to differentiate towards a more TE state and become largely depleted by memory time points, while

clones with comparatively lower signalling were memory-like and able to persist after antigen clearance. Interestingly, it seems that stronger TCR signalling was associated with higher expression of T_H1 markers, while weaker TCR signals correlated with higher expression of T_{FH} markers,⁴⁴ suggesting that there may be a connection between lineage differentiation and memory potential for CD4⁺ helper T-cells.

Utilizing influenza A virus (IAV) as an infection model, McKinstry *et al.*⁴⁵ showed that IL-2 is crucial at a late checkpoint for effector helper T-cells to survive the contraction phase, allowing for the transition into resting memory cells. To circumvent defects in initial T-cell priming caused by IL-2 deficiency, the authors first activated CD4⁺ T-cells *in vitro* with exogenous IL-2 and then transferred these cells into naive mice for infection. Following IAV challenge, both *in vitro* primed wild-type and IL-2-deficient donors showed similar cell numbers at the peak of infection and production of IFN γ ; however, the IL-2-deficient population quickly declined and was undetectable by day 28 of infection. Exogenous administration of IL-2 during days 5–7 of infection successfully restored memory cell numbers for IL-2-deficient CD4⁺ T-cells, demonstrating the importance of IL-2 for CD4⁺ memory T-cell generation in this context. Furthermore, a recent study by DiToro *et al.*⁴⁶ with LM infection showed that as early as 20 hr after antigen exposure *in vivo*, IL-2 production in CD4⁺ T_H effector cells strongly correlated with T_H fate differentiation during infection, again supporting a link between lineage specification and memory formation. To further highlight the importance of IL-2 in T_H memory, Shakya *et al.*⁴⁷ identified a role for TF Oct1 and its coactivator OCA-B in poising the *Il2* locus for robust expression in memory CD4⁺ T-cells, unveiling an important mechanism by which memory CD4⁺ T-cells control IL-2 production. However, these studies regarding TCR signalling and IL-2 in CD4⁺ memory T-cells were done without investigation of specific T_H lineages. Therefore, further investigations into the required transcriptional and epigenetic regulation for generation and maintenance of memory T_H subsets are needed. Additionally, while targeting peripheral memory cells in vaccination strategies can provide systemic protection, in some cases a localized strategy in which tissue-resident cells at barrier surfaces are activated as front-line defense against recurrent infections may be more effective, thus how these signals pertain to T_{RM} will be informative.⁴⁸

Tissue-resident CD4⁺ memory T-cells

Much like circulating CD4⁺ memory T-cells, studies of tissue-resident lymphocytes have predominantly focused on CD8⁺ T_{RM} due to the heterogeneity of CD4⁺ memory T-cells and the existing gaps in knowledge regarding mechanisms governing memory CD4⁺ T-cell formation.

Nevertheless, recent studies have highlighted a prominent population of long-lived CD4⁺ T-cells within many non-lymphoid tissues, including the lungs,^{36,49–57} small intestine (SI),^{12,18,58–60} skin,^{15,61–64} and female reproductive tract (FRT)^{18,65,66} (Fig. 2). Similar to their CD8⁺ T-cell counterparts, CD4⁺ T_{RM} have been shown to facilitate rapid immune defense upon re-exposure to antigen and can supplant innate immunity in recognizing and responding to recurrent infections.⁶⁷ However, much remains to be explored about the phenotype, function and maintenance of CD4⁺ T_{RM} during infections. Additionally, differences exist between CD4⁺ and CD8⁺ T_{RM} in tissue localization, surface marker expression and cytokine cues driving T_{RM} formation; these outstanding questions in the field must be addressed to better define the identity and differentiation of CD4⁺ T_{RM}.

Classically, tissue-resident memory T lymphocytes have been defined using parabiosis experiments in which a naive mouse and an immune mouse, previously exposed to antigen, are surgically joined to create a shared circulatory system.^{56,68} Thus, all circulating cells will normalize between both partners while the non-circulating tissue-resident cells remain lodged in the tissues of the immune mouse. Alternative methods have been developed and validated to assess whether cells remain in tissues, including intravenous injection of a fluorescently labelled antibody to mark cells in the circulatory system.⁶⁹ Any cells positive for the label are considered 'circulating', while unlabelled cells are assumed to have limited access to circulation and are therefore 'tissue-resident'.⁵⁶ To determine the protective functions of tissue-resident lymphocytes in secondary infection, immune mice are treated with FTY-720, an agonist of sphingosine-1-phosphate receptor 1 (S1PR1), which causes decreased surface expression of S1PR1 and therefore prevents egress of circulating memory cells from lymph nodes.⁷⁰ When these mice are re-challenged with the original pathogen, any immune response at the local site of infection will be mediated only by cells resident to that tissue.⁷¹

Lung CD4⁺ T_{RM} cells

The lungs contain a population of CD4⁺ T_{RM} that have been shown to play a critical role in recruiting CD8⁺ T-cells and enhancing secondary immune responses against bacterial, viral and worm infections.^{51,55,56,72} In an influenza infection model, antigen-specific memory CD4⁺ T-cells migrate to the lungs and are retained in the tissue without recirculation, as demonstrated by parabiosis experiments.⁵⁶ This subset of CD4⁺ T-cells shows a distinct phenotype from circulating populations; specifically, high expression of CD69, a membrane-bound type II C-lectin receptor and known marker of tissue retention.^{73,74} Functionally, these lung-resident CD4⁺ T-cells provide protection from influenza virus when transferred

to naive mice. Similar to its role in directing long-term memory fate as discussed above, IL-2 also supports the formation of antigen-specific lung CD4⁺ T_{RM} with a transcriptional signature distinct from that of circulating CD4⁺ T-cell populations but similar to that of CD8⁺ T_{RM}.^{51,75} Interestingly, Strutt *et al.*⁵¹ also identified an IL-2-independent population of influenza-specific lung CD4⁺ T_{RM} following infection, suggesting that IL-2 may not be the only cytokine regulating lung T_{RM} development and maintenance. In fact, IL-15 was shown to be essential for these IL-2-independent cells, acting as an 'alarm' at local sites of infection to promote both CD8⁺ T-cell responses and induce long-lived CD4⁺ T_{RM}.^{42,51} Using fluorescently labelled antibody injection, Hondowicz *et al.*⁴⁹ showed that LCMV-specific CD4⁺ T-cells migrated to the lungs as a Tbet^{hi} T_H1 subset with CD69 expression similar to influenza experiments. Again, the establishment of LCMV-specific lung T_H1 T_{RM} cells required IL-2 signalling; however, for long-term maintenance and survival, additional interactions with B-cells in tissues were necessary.⁴⁹

Several groups have shown that allergens in an asthma model also elicit CD4⁺ T_{RM} responses in the lung. After exposure to HDM, allergen-specific CD4⁺ T_{RM} in the lungs were shown to be resident by parabiosis experiments.³⁶ These cells expressed high levels of CD69 and required IL-2 signalling for their migration to the lungs, similar to the studies above with viral pathogens. Interestingly, the TF Bcl6, the fate-determining factor for T_{FH} cells, prevented HDM-specific CD4⁺ T_{RM} from entering the lungs, and loss of Bcl6 actually increased the T_{RM} population in the tissue. These results suggest a possible antagonistic relationship between T_{RM} and memory T_{FH} cells, and highlight a role for TFs in directing the development of tissue-resident populations. In another study using the HDM model, lung CD4⁺ T_{RM} clustered around the airways at rest and rapidly reactivated upon secondary exposure within their clusters to produce IL-4, IL-5 and IL-17 and recruit dendritic cells.⁵⁰ Additional studies highlight a role for IL-7, along with IL-2, in the maintenance of allergen-specific CD4⁺ T_{RM} cells in the lung parenchyma and airways. However, these IL-7-dependent CD4⁺ T_{RM} did not express CD69,⁵² suggesting either that CD69 may not be a conclusive marker for CD4⁺ T_{RM} or that CD4⁺ T_{RM} are highly heterogeneous, and further studies must focus on elucidating the different subsets. Additionally, in both viral infection and allergen-induced asthma models, it remains unclear whether the same cells can become resident in both the parenchyma and airways, and if similar survival signals sustain such subsets.

Skin CD4⁺ T_{RM} cells

A second well-studied tissue for CD4⁺ T_{RM} cells is the skin particularly in a herpes simplex virus (HSV)

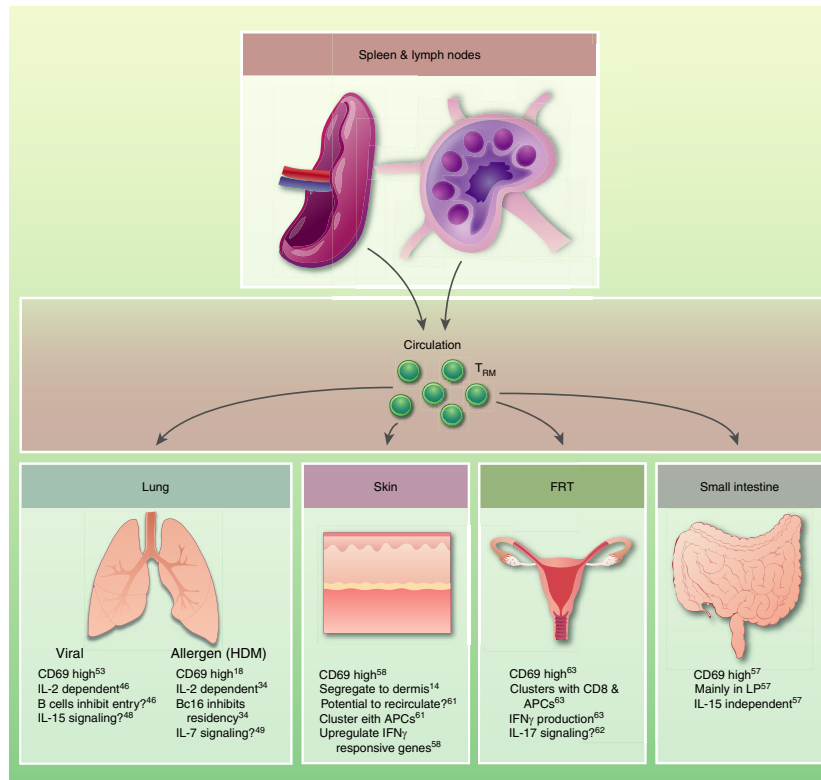


Figure 2. Following infection, CD4⁺ tissue-resident memory cells (T_{RM}) are recruited to tissues from circulation and secondary lymphoid organs (SLOs). The majority of CD4⁺ T_{RM} express high levels of CD69 and can form clusters with other resident immune subsets, including CD8⁺ T_{RM}, macrophages and antigen-presenting cells (APCs). Cytokine signalling may also play a role in recruitment and retention, although specific cytokines are preferred by each tissue.

infection model. Initial studies by Gebhardt *et al.*¹⁵ using intravital microscopy demonstrated that HSV-specific gDT-II CD4⁺ T-cells showed a migration pattern distinct from that of CD8⁺ T-cells in the skin following infection, homing to the dermis as opposed to the preference of CD8⁺ T-cells for the epidermis. Additionally, dermal T_H cells were significantly more motile and had lower expression of CD103 compared with CD8⁺ T-cells. Thus, it appears that CD4⁺ T_{RM} can migrate between the skin and circulation much more easily than CD8⁺ T_{RM} and may not be a fully resident population. In another study of skin HSV response, Collins *et al.* examined skin from naive mice and found resting CD4⁺ T-cells preferentially clustered around hair follicles. Upon infection, circulating CD4⁺ T-cells were rapidly recruited to the skin, specifically to the perifollicular regions; the majority of recruited cells were lost by day 30, but a small percentage survived in tissue long after initial antigen exposure.⁶⁴ These skin CD4⁺ T_{RM}, as demonstrated by parabiosis experiments, also aggregated in APC-associated clusters, consistent with experiments done in the FRT,^{66,76} as discussed below. Additional studies of skin CD4⁺ T_{RM} following *Leishmania major* (*L. major*) infection showed evidence of a *L. major*-specific resident population in the dermis with increased expression of IFN genes and chemokine

signalling pathways.⁶¹ These CD4⁺ T_{RM} cells were able to control parasite growth following a secondary challenge, presumably through the aforementioned IFN response and recruitment of circulating immune cells. Through FTY-720 treatment experiments, resident CD4⁺ T-cells were found to be the main contributors to protection against *L. major*, recruiting inflammatory monocytes for production of reactive oxygen species to kill the parasites.⁶² These results highlight another function of CD4⁺ T_{RM} in enhancing secondary responses and protection against recurring pathogens.

CD4⁺T_{RM} cells in other mucosal tissues

Through parabiosis experiments, CD4⁺ T_{RM} have been shown to be active in the FRT in response to HSV-2 infection.^{66,77} Intravaginal HSV-specific CD69⁺ CD4⁺ T_{RM} localized to memory lymphocyte clusters containing CD8⁺ T-cells, macrophages and other APCs, and were maintained in these structures by chemokines secreted by macrophages. Upon a secondary re-challenge with lethal HSV-2, resident T_H cells alone were sufficient for protection, largely mediated by CD4⁺ T_{RM} production of IFN γ to inhibit viral replication. In this context, FRT-resident CD4⁺ T-cells were more directly involved in clearing the

infection as opposed to their classic role in alarming and recruiting circulating populations.

The SI contains a potential subset of tissue-resident CD4⁺ T-cells, though no parabiosis experiment has been performed to definitively characterize this population as resident. In general, intestinal helper T-cells can be found as both intraepithelial lymphocytes (IELs) and lamina propria lymphocytes (LPLs) with a greater proportion of cells in the LP. The majority of studies of gut-resident CD4⁺ T-cell subsets have focused on endogenous polyclonal IELs that originate from the circulating population.^{59,78,79} Upon entering the SI, these CD4⁺ IELs downregulate the TF ThPOK responsible for thymic CD4⁺ T-cell fate and upregulate Runx3, the fate-determining factor for differentiating CD8⁺ thymocytes. This switch in programming towards a more cytolytic function appears to be important in the CD4⁺ T-cell response to endogenous gut microbiota.⁷⁸ Nevertheless, this study characterizes polyclonal CD4⁺ T-cells with variable TCR specificity and affinity, and further investigations are necessary to determine whether similar transcriptional changes are seen in antigen-specific responses. In an LM infection, antigen-specific CD4⁺ T-cells preferentially migrated into the LP (though a small population was seen in the IEL) to form a long-lived antigen-specific memory population that was predominantly T_H1, expressed high levels of CD69 and, unlike lung-resident CD4⁺ T-cells, was independent of IL-15 signalling.⁶⁰ Interestingly, this gut 'T_{RM}' population had low Ly6C expression relative to other lymphocytes in circulation, similar to the MP population of circulating T_H1 cells discussed in the T_H1 and T_{FH} CD4⁺ memory T-cells above. This suggests that circulating and resident memory cells may share a common precursor.

As discussed in the studies above, CD4⁺ T_{RM} differ greatly between infection models and tissues, although some general principles can be drawn (Fig. 2). As is the case with CD8⁺ T_{RM}, the majority of CD4⁺ T_{RM} express high levels of CD69, but CD103 expression varies within and between tissues.^{15,56,60,66} Across multiple tissues – lung, skin, FRT – CD4⁺ T_{RM} form clusters with other resident immune subsets including CD8⁺ T-cells, macrophages and APCs.^{52,64,66} These clusters position CD4⁺ T_{RM} in close proximity to the cells they need to activate in case of a recurrent infection. This may be an optimized way for helper T_{RM} cells to perform their 'sense-and-alarm' function,⁶⁸ initiating the innate response to recruit circulating cells and activating CD8⁺ T-cells to fight off pathogen. Additionally, cytokines appear to be critical in the recruitment, formation and maintenance of CD4⁺ T_{RM} in many tissues.^{50–52,60} However, which cytokines are important for which tissues remain unknown, although IL-2 may play a key role for all T_{RM} given its known effects in circulating memory populations.⁸⁰ It is also unclear which

CD4⁺ T_H effector subset, if any, gives rise to T_{RM}, or whether the tissue-resident precursor is distinct from the peripheral population completely, residing in the tissue until the right signals are detected to induce differentiation.

Conclusion

The diversity and plasticity of effector CD4⁺ T-cells create a heterogeneous memory pool, making the study of helper T-cell memory differentiation complex. While there are some promising markers to differentiate between memory T_H1 and T_{FH} memory subsets,^{24,27} it is still unclear whether both helper memory populations originate from their respective effector cells or whether the 'stem-like' properties of T_{FH} cells make them the primary precursor.²⁵ Likewise, in other infection systems that elicit T_H2 or T_H17 effector cells, we do not know how these effector populations contribute to the final pool of memory cells. Adding further to the complexity, CD4⁺ T_{RM} cells are highly variable across tissues, and different cues drive their establishment and retention. It is unclear whether the precursor for CD4⁺ T_{RM} cells originates from the same memory-precursor population that yields circulating memory cells or if it is found even earlier, before the effector versus memory decision. T_{FH} cells may also contribute to the CD4⁺ T_{RM} pool, although currently no evidence exists for a T_{FH}-like subset in tissues. Lastly, it is clear that surface markers do not accurately identify subpopulations in effector and memory pools, and further work requires examining TFs and regulators that may direct the memory programme. One possible approach to parse the heterogeneity of CD4⁺ memory T-cells is through bulk and single-cell epigenetic and transcriptional profiling of cells in the circulation and tissues over the course of an infection to identify whether an early memory precursor exists or whether memory potential is programmed as effector cells contract and die.^{75,81,82} Paired with adoptive transfers of putative subsets, it will be possible to identify key factors at each step in the effector-to-memory transition, and in the formation and survival of the tissue-resident subset.

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Disclosures

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